FORM PTO-1390 (REV 11-2000)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1303-122

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U S APPLICATION NO. (If known, see 37 C F.R 1 5) 09/831820 Unknown

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP99/08847	17 November 1999	17 November 1998

TITLE OF INVENTION

METHOD FOR THE QUANTITATIVE DETECTION OF NUCLEIC ACIDS

APPLICANT(S) FOR DO/EO/US

LOCATELLI et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information. PTO-1449/ International Search Report

U.S. APPLICATION NO (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/831820 Unknown	PCT/EP99/08847	1303-122

21. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):

-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $860.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO $710.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $690.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

CALCULATIONS PTO USE ONLY							
ENTER APPROPRIATE BASIC FEE AMOUNT =						$	860.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	130.00
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE			
Total Claims	18	-20 =	0	X	$18.00	$	0.00
Independent Claims	3	-3 =	0	X	$80.00		0.00
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				$270.00		$	270.00
TOTAL OF ABOVE CALCULATIONS =						$	**1260.00**
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.							0.00
SUBTOTAL =						$	**1260.00**
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00
TOTAL NATIONAL FEE =						$	**1260.00**
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +						$	0.00
Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00)						$	0.00
TOTAL FEES ENCLOSED =						$	**1260.00**
						Amount to be: refunded	$
						Charged	$

a. ☒ A check in the amount of $1260.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.

d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

NIXON & VANDERHYE P.C.
1100 North Glebe Road, 8th Floor
Arlington, Virginia 22201-4714
Telephone: (703) 816-4000

SIGNATURE

Arthur R. Crawford
NAME

25,327 REGISTRATION NUMBER

May 15, 2001 Date

524196

METHOD FOR THE QUANTITATIVE DETECTION OF NUCLEIC ACIDS

The present invention relates to a method for quantitative detection of nucleic acids from a biological fluid sample.

The method of the invention may be suitably applied to diagnosis of viral and any other pathogenic agents and to monitor safety and/or genetic composition of waters, foods and plant species used in the alimentary field.

BACKGROUND ART

A commonly used strategy to detect the presence of pathogens, in biological fluids, is the detection of an antigen (direct method) or a respective antibody (indirect method). However, this strategy, performed with immunometric techniques such as ELISA, IFA or Western Blotting, is limited because of the scarce quantitation accuracy, precision and sensitivity, of the different antibody cross-reactivity and of the impossibility to obtain precocious diagnosis.

Another approach relies on the detection of nucleic acids specific for each kind of molecular target from any biological source, using the amplification by polymerase chain reaction (PCR). This technique, in its more sophisticated version i.e. the quantitative competitive PCR (qcPCR), makes it possible to reach a high sensitivity and a quite accurate quantitative measure, as well as to obtain a diagnosis a short time after contact between the patient and the pathogen. Nevertheless the precision and accuracy of this system is assured in a narrow quantitation range, thus forcing the operator to multiply the number of replies (typically 8) of the sample under investigation; furthermore a long time and additional costs

for the amplified product detection steps are necessary.

The first systems that assessed PCR kinetics in real time were based on an intercalating substance such as ethidium bromide. This substance binds to the polymerizing double strand DNA proportionally, enhancing its fluorescence in response to UV excitation; the fluorescence emitted from the intercalated ethidium molecules was registered by a CCD camera in a thermal cycler equipped to irradiate the samples with UV rays and plotted against the amplification cycle number (Higuchi et al., Biotechnology 10:413-417). The main limitation of this technique is that the signal is generated also from the unspecific PCR products.

Subsequently the method known as TaqMan, described in US 5210015 was introduced. This method is based on the real time detection of the fluorescence deriving from the degradation, directly dependent on the nascent PCR product, of a labeled probe specifically hybridizing to the segment to amplify, by means of the Taq polymerase enzyme. The PCR reaction mix contains a non-extendable oligonlucleotide probe, labeled with two fluorescent molecules, a reporter at the 5' end, and a quencher at the 3' end; the probe sequence must be complementary to a region of the DNA under investigation located between the two annealing sites of the oligonucleotide primers.

During the PCR amplification reaction, the Taq Polymerase enzyme specifically activated by the primers starts duplicating the DNA under investigation; when the enzyme contacts the probe annealed to the DNA, cuts it by its 5' nuclease activity, removing it and consequently separating the fluorescent molecules; the emission from the reporter fluorochrome becomes thus measurable and, each DNA molecule being accompanied by a reporter molecule release, the total fluorescence is at any time

proportional to the amplified DNA amount. The Sequence Detection System 7700 ABI PRISM (produced and distributed by Perkin Elmer) can work both as a DNA amplifier and a collector of fluorescence signals from samples during the PCR reaction. These signals are then processed by a software capable of extrapolating the starting DNA amount in the analyzed samples by a standard curve built with the fluorescence signals from samples with known DNA content. It must be noted that such a system is endowed with two specificity levels: the specific annealing of the primers and the specific annealing of the probe.

SUMMARY OF THE INVENTION

A method generally applicable to the nucleic acid quantitation techniques based on the polymerase and 5' nuclease activity of nucleic acid polymerases, improving the efficiency of the techniques themselves, along with an enhanced sensitivity, accuracy and precision and a reduced measure variability, has now been found.

The method of the invention is based on the use of a calibrator during the steps of extraction of the sample, of target nucleic acid amplification and subsequent detection with suitable probes able to differentially hybridize to the calibrator and the target sequence.

The method of the invention can be applied to any absolute quantitation of nucleic acids from different biological sources, for example to the quantitation of viral or bacterial pathogens in body liquids (liquor, urine, plasma, serum, synovial fluid) or to the quantitation of environmental or food contaminants.

DETAILED DESCRIPTION OF THE INVENTION

Nucleic acid quantitation techniques, using the polymerase and 5' nuclease activity of the nucleic acid polymerases, rely on the extraction of

nucleic acid from the sample, on the preparation of a reaction mix containing a polymerase, primers specific for the target sequence and a probe specific for a target sequence included between the regions complementary to the two primers, said probe being labeled with a reporter (this reporter being preferably a fluorochrome) and a quencher, followed by determination of the signal from the reporter label released when the polymerase contacts the 5' end of the probe annealed to the target nucleic acid.

According to the method of the invention, a known amount of the template nucleic acid, hereafter referred to as calibrator, is added to the sample before the extraction of the nucleic acid to be quantitatively detected (target nucleic acid). The calibrator has a sequence identical to the target nucleic acid sequence with the exception of one or more regions whose sequence is different from the corresponding regions of the target nucleic acid, having with respect to the latter a randomized sequence and a similar Tm. More specifically, one of those different regions must be the region complementary to the probe, with a Tm comprised in the range of ±4°C the Tm of the target nucleic acid, preferably in the range ±2. The other different regions of the calibrator may be those annealing with the primers, which have randomized sequences with respect to the target nucleic acid and a Tm comprised in the range of ±1°C the Tm of the target nucleic acid.

When this second embodiment is provided, i.e. when the calibrator regions complementary to the primers are varied with respect to the target nucleic acid, in addition to the regions complementary to the probe, it is possible to extremely reduce the competition events occurring between the target template and the calibrator, and to simultaneously measure the

target template and the calibrator in a single reaction tube.

After extraction, the primers, and separately the probes derivatized with a reporter and a quencher, where the reporter can be equal or different among different probes, are added to the extracted sample-calibrator mixture. Two or more primers may be added to the reaction mixture, depending on the number of variable regions present on the calibrator annealing with the primers. Preferably, up to 3 pairs, more preferably up to two pairs of primers (forward and reverse) are used. Furthermore a thermostable polymerase with 5'-3' nuclease activity is added, thus starting the polymerase/nuclease reaction.

The reaction is carried out in a Sequence Detection System 7700 ABI PRISM that can work both as a DNA amplifier and a collector of fluorescence signals emitted from the reporter markers released upon polymerase nuclease activity. Virtually, three reactions are carried out in parallel, one of them in the presence of target nucleic acid specific probe, one in the presence of the calibrator specific probe/primers, and one in the presence of both.

The reaction in the presence of the target nucleic acid specific probe permits quantitation of the copy number of the extracted target nucleic acid (N_o). The reaction in the presence of the calibrator permits quantitation of the calibrator copy number recovered upon extraction (C_o). The reaction in the presence of both permits calculation of the total number of target templates and calibrator (T).

It is thus possible to calculate the percentage of the calibrator recovery yield R:

$$R = C_o/C,$$

from which the calibration factor (cal)

$$cal = 1/R$$

and thus the actual number of nucleic acid units in the sample before extraction are obtained:

$$N = N_o \times Cal$$

The relation

$$T = N_o + C_o$$

assures that the amplification efficiency of the standard and calibrator DNA remain identical.

The calibrator non-amplification makes it possible to detect any false negatives (technical errors or presence of inhibitors), which represent one of the most important drawbacks when using amplification methods in clinical diagnostics.

The reaction conditions are the same as those commonly adopted in qcPCR reactions (Petrik j. et al., J. Virol. Methods, 64:147-159, 1997). The reaction conditions can be modified in order to compensate for the competition events, more exactly the primer concentration, the polymerase enzyme concentration, the annealing/extension time, or the concentration of cofactors such as $MgCl_2$ can be modified.

The target nucleic acid can be DNA or RNA, preferably DNA, while primers and probes are preferably deoxyribonucleotide sequences. When the nucleic acid is RNA a previous retro-transcription step is required in order to obtain the corresponding DNA. The probes include a fluorescent reporter label and a quencher label to reduce or to avoid fluorescence from the reporter label when the probes are free in solution.

TET (tetrachloro-6-carboxy-fluorescein), JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein), HEX (hexachloro-6-carboxy-fluorescein) are examples of reporter, preferably TAMRA (6-carboxy-tetramethyl-

rhodamine) and FAM (6-carboxy-fluorescein) are used as quencher and reporter, respectively.

Preferably the 5' end of said probes is in the range of 1 to 30 nucleotides from the 3' end of the forward primer, i.e. at a distance to permit the reporter label release in the absence of nucleic acid polymerization. Preferably, the probes have a blocked 3' end in order to prevent the extension by polymerase, have a Tm higher than the primer Tm, and comprise 18 to 30 nucleotides.

The nucleic acid polymerase is a thermostable polymerase with 5'-3' nuclease activity, preferably a DNA polymerase, and more preferably a DNA polymerase derived from the Thermus species.

A great advantage of the described method is represented by the possibility to detect samples containing inhibitors and to mathematically characterize the yield of genetic material extraction. A further advantage is represented by the possibility of simultaneously measuring the calibrator and the target nucleic acid in the same reaction tube. The possibility of detecting total inhibitors of amplification reaction from the samples allows the elimination of false negatives often occurring with known techniques. Moreover, the calibration does not necessitate further standardization which is conversely required, for example, by the TaqMan technique (see Chatellard P. et al., J. Virol. Methods, 71:137-146, 1998).

The present invention also provides a kit to perform the above described method, including, depending on the target nucleic acid, a suitable calibrator, a probe specific for the target nucleic acid, two or more primers and a nucleic acid polymerase.

According to a preferred embodiment, the method of the invention is employed to quantitatively detect the genomic nucleic acid of the HHV-

6, HHV-7, HHV-8, and HIV viruses, in a sample.

Particularly, in the case of HHV-6, the assay is divided into two steps: in the first step gene extraction is performed by a method based on the standard lysis-purification protocol by phenol-chloroform; in the second step the amplification reaction is carried out with specific primers and probes, selected by the "Primer Express" software (Perkin Elmer).

The primer and probe have been designed to amplify both type A and type B HHV-6 strains with the same efficiency. This feature is responsible for very high diagnostic sensitivity.

The calibration according to the method of the invention relies on adding to the sample, before DNA extraction, a known amount of a template DNA amplifiable with the same kinetic properties as the HHV-6 amplicon, but at the same time clearly distinguishable from it. A DNA fragment identical to that amplified by the HHV-6 primers, apart from the region complementary to the probe that has been modified so as to preserve the same nucleotide composition but with a random sequence, and with the same melting temperature (Tm), is thus cloned; the obtained plasmid, termed "calibrator", is amplified by the same primers and with the same kinetic as the standard plasmid, but it is revealed by the 7700 software only if the PCR mix contains a probe with a sequence complementary to the random sequence.

The calibrator plasmid is expanded and accurately quantitated by the spectrophotometer so as to add an exact amount of it to the samples to be extracted. Upon DNA extraction, samples thus contain a certain number of HHV-6 genomes and calibrator plasmid copies, dependent on the total yield of such an extraction; the HHV-6 DNA quantitation is thus possible, assuming such a yield to be identical for both the molecular

species.

The reaction specific conditions are indicated in further detail in the example section.

To compensate for competition events, the reaction conditions were modified as follow: the primer concentration was elevated ten-fold, enzyme concentration was doubled, the amplification annealing/extension was increased by 8 seconds per cycle starting from the initial 60 seconds. However the competition events between templates narrowed the range of absolute quantitation (calibrated) from 7 to 5 order of magnitude, 2 above and 2 under the value of the calibrator input, while the relative quantitation range (non calibrated) and the ability to detect the experimental false negatives are unchanged.

According to an alternative embodiment, the original calibrator sequence has been further randomized in the two primers coding regions maintaining the theoretical Tm of the two original primers (as calculated by the specific software Primer Express PE foster city CA). The relevant sequences of the new calibrator molecules are reported in Table 2 of the examples. The target DNA molecule and the calibrator were amplified with the same kinetics, as evidenced by the following equations:

$y=37.804 + -3.4402 \times LOG(x)$ $R = 1.000$ for the standard template amplification, and $y= 38.543 + -3.568 \times LOG(x)$ $R = 0.998$ for the calibrator template amplification.

The two molecules added in the same tube were correctly co-amplified, using standard PCR reactions, for more than 7 order of magnitude with no necessity to compensate for competitive events.

In order to carry out the simultaneous detection and quantification of target DNA and calibrator in a single tube the calibrator was derivatized at

the 5'end with VIC (Pe Biosystem), a fluorescent molecule having an emission spectrum different from the one used for the target DNA molecule detection.

To eliminate the interference generated by the partial overlapping of the emission spectra of the two dyes, the calibrator PCR conditions were modified by reducing the primers and probe concentrations (final concentration 50nM for both). The reduction of the emission signal of VIC reporter was achieved without modification of the Ct (Cycle threshold) value, thus allowing a reproducible quantitation of the calibrator itself.

The spectral interference was completely avoided by adding the calibrator in a fixed concentration one log higher than the maximal amount of standard used in the reference curve (i.e. 10,000,000 copies of calibrator for a standard curve in which the highest concentration of standard was 1,000,000 copies reaction).

The following examples illustrate the invention in further detail.

EXAMPLES

Example 1

Selection of the HHV-6, HHV-7, HHV-8, HIV, 35s CAMV and Mycobacterial sequences.

The U67 HHV-6 region (A variant, GeneBank Accession N°. X83413) and the 26 HHV-8 orf region (Chang et al., Science 266, 1865-1869, 1994) the HHV-7 region (Gene Bank AF037218), 35sCAMV (Gene Bank AF140604), RegX-SenX (Gene Bank MTY 13628) and 156110 (Gene Bank X17848), and the HIV region mapping between the LTR and gag region from nucleotide 684 to 810 using the sequence HXB2CG-Accession number K03455 (Gene Bank) are reported in Table 1 (primers

and probe of the target nucleic acid and calibrator probe). The HHV-6 sequences (primers and probe for the target, calibrator's primers and probe) are reported in Table 2.

The probe sequences of the calibrators (HHV-7, HHV-8, HIV-1, CAMV, Myc. T.) and the calibrator's primers (HHV-6) were designed randomizing the probe region of the standard, while maintaining:

1. the same base composition (G+C/A+T) of the standard
2. an identical Tm (calculated by Perkin Elmer software)
3. an identical length (in order to have the same amplification efficiency)
4. and being characterized by an absolute absence of homology with the standard (target) in order to avoid cross-hybridization and interferences.

Table 1

HHV-7	Primers	Probes
	Forward 5'AGCGGTACCTGTAAAATCATCCA3'	Standard 5' ACCAGTGAGAACATCGCTCTAACTGGATCA 3'
	Reverse 5' AACAGAAACGCCACCTCGAT 3'	Calibrator 5' TAAGCCCTGACCGCACGGGTATAATACTAA 3'
HHV-8	Primers	Probes
	Forward 5'GTCCAGACGATATGTGCGC3'	Standard 5'CATTGGTGGTATATAGATCAAGTTCCGCCA3'
	Reverse 5'ACTCCAAAATATCGGCCGG3'	Calibrator 5'ACTATTCCATGCGGAATTCGAGCATAGTTG3'

(continued)

Table 1 (continued)

HIV-1	Primers	Probes
	Forward 5' TACTGACGCTCTCGCACC 3'	Standard 5' ATCTCTCTCCTTCTAGCCTCCGCTAGTCAA 3'
	Reverse 5' TCTCGACGCAGGACTCG 3'	Calibrator 5' ACTCTCAGCGGCATTCTCCTCACTTCTACT 3'
CAMV	Primers	Probes
	Forward 5' GTCTTGCGAAGGATAGTGGGA 3'	Standard 5' TGCGTCATCCCTTACGTCAGTGGAGAT 3'
	Reverse 5' CACGTCTTCAAAGCAAGTGGA 3'	Calibrator 5' ATCGCTACATGCTAGGCATCTGTGTGC 3'

(continued)

Table 1 (continued)

	Primers	Probes
Myc. T. 1	Forward 5' AGGAGGAGTGGCGCTGATG 3'	Standard 5' ACGAGGAGTCGCTGGCCGATCC 3'
	Reverse 5' ACTCGGCGAGAGCTGCC 3'	Calibrator 5' TCCAGCGTCAGGCGTAGGCAGC 3'
	Primers	Probes
Myc. T. 2	Forward 5' AGGCGAACCCTGCCCAG 3'	Standard 5' TCGACACATAGGTGAGGTCTGCTACCCACA 3'
	Reverse 5' GATCGCTGATCCGGCCA 3'	Calibrator 5' ACTACGACTACGGCTGCGATCGACATCGAT 3'

Table 2

HHV-6	Primers	Probes
	Forward 5' CAAAGCCAAATTATCCAGAGCG 3'	Standard 5' CACCAGACGTCACACCCGAAGGAAT 3'
	Reverse 5' CGCTAGGTTGAGGATGATCGA 3'	Calibrator 5' TACGCAACGCCAACAGACCTAGCGA 3'
	Calibrator	
	Primer forward 5' CCGGAAACCGAACATTACTGAA 3'	Probe 5' TACGCAACGCCAACAGACCTAGCGA 3'
	Primer reverse 5' TTACGTGAGGATGATCGAGGC 3'	

Example 2

HHV-6 Standard (target nucleic acid) and Calibrator cloning and preparation.

The fragments used for the standard and calibrator DNA construction in the HHV-6 virus detection system are schematically represented in Figure 1 (1-A and 1-B respectively).

The Standard fragment sequence was obtained by amplification of the viral DNA from HHV-6 GS strain and subsequent cloning into pCRII plasmid vector (Invitrogen). The calibrator fragment (133 bp) was chemically synthesized by an Oligosynthetizer (Perkin Elmer), and then cloned in the same vector as above.

After cloning, both fragments were completely sequenced in order to identify: i) the identity (co-linearity) of the standard fragment with the original viral DNA, ii) the identity of the calibrator fragment with the artificially designed sequence.

As shown in Figure 1, the arrows, oriented in the transcription direction, indicate the oligonucleotide sequence employed as primers. The dotted line identifies in both constructs the regions of 25 nucleotide used as the probes (lowercase) differentiating the two constructs, otherwise identical in the remaining 108 nucleotides. These regions, even though they have the same base composition and a very similar Tm, were designed in such a way as to function as a heterologous system, allowing avoidance or minimization of the cross-hybridization events between the probes employed in the specific fluorimetric detection and the standard and calibrator fragments.

Example 3

HHV-6 calibrator/standard system validation

Absence of cross-hybridization

We thus experimentally verified the absence of spurious signals from cross-hybridization. Increasing concentrations of the standard and calibrator fragment (from 101 to 106 plasmid copies per PCR reaction) were measured using the homologue probe (standard probe for the standard DNA, calibrator probe for the calibrator DNA) or the heterologous probe (standard probe for the calibrator DNA, calibrator probe for the standard DNA). Figure 2B shows the detection of the various template amounts employing the probe homologous to the fragment to measure, where: a) indicates the standard amplification curve and, b) the calibrator curve. Detection takes place for both constructs with overlapping kinetics (curves 1-5, a, b) furthermore indicating that the template amplification is proportional to the measured fluorescence signal. The use of heterologous probe (Fig. 2A) for both templates does not generate a signal appreciably higher than the system background noise.

Co-linearity

Amplification co-linearity of the two templates was measured by comparing the two regression line equations generated from the values obtained as threshold cycles as a function of the increasing copy number of the employed template.

The resulting equations are:

y=37.804 + -3.4402 X LOG(x) R = 1.000 for the standard template amplification

y= 38.543 + -3.568 X LOG(x) R = 0.998 for the calibrator template

amplification.

The slope ratio of the two lines is 1.017 thus indicating that the two systems are perfectly overlapping both as amplification efficiency and as fluorimetric detection dynamics.

Calibration range

Amplification of the two templates in the same PCR reaction causes an appreciable modification of the amplification curve detected by the system (Fig. 3A). In fact it is possible that for increasing copy amounts, i.e. 500 calibrator copies in unique dose co-amplified in the presence of 0, 500, 5,000, 50,000, 500,000 standard copies (from 1a-1e respectively), the fluorescent signal is changed both in the accumulation kinetic and in the final amount of the released product. At the threshold cycle (A' insert), standard template concentrations equivalent to or higher by a Log (A' insert – 1a-1c curves) do not influence the accuracy of the calibrator quantitation. For higher concentrations (1d, 1e curves) the quantitation is partially (marked delay of the threshold cycle, 1d) or completely impaired (1e). The optimization of the PCR conditions, and particularly the combined actions of primer concentration increase (from 300 nM to 3 μM), doubling the enzyme concentration (from 0.625 to 1.25 AmpliTaq Gold units), and the increase in length of every PCR cycle (8 sec. increment per cycle during the annealing and extension steps), cause an improvement of the amplified product accumulation kinetic and of the final yield of the fluorescence signal (Fig. B, curves 1a-1e). At the threshold cycle (B' insert), standard template concentrations up to 2 Log (50,000 copies) higher than the calibrator input, do not modify its quantitation (B' curves 1e-1d). For higher concentrations (500,000 copies) the fluorimetric signal from the calibrator probe is measurable, although

the lack of signal exponential increase does not allow maintenance of an accurate measure of the calibrator (B' curve 1e).

In opposite conditions (Fig. 4A), i. e. with excess of calibrator up to 2 Log concentration with respect to the standard, (e. g. 500 copies of calibrator vs. 5 copies of standard), the analysis of the reaction kinetics is as above. In particular, in optimized conditions, the quantitation at the threshold cycle (A' insert; "a" indicates the amplification curve of the standard in the absence of the calibrator, "b" the curve measured in the presence of the calibrator), in this case of the standard, is not modified (A' curves 1a-1b: 100 copies of standard and 25 copies of standard concentration, 3a-b:5 copies of standard concentration).

By means of this optimization it can thus be obtained an accurate quantitation of a template whose amount is unknown, with the following practical advantages:

1) Absolute control (both qualitative and quantitative) of the whole process of sample purification and amplification with a dynamic range of at least 5 Log (e. g. 5 to 50,000 copies per reaction);
2) Quantitative control of purification and amplification process with a dynamic range of at least 7 logarithms (false negatives due to technical error or to the presence of contaminants inhibiting the PCR reaction).
3) Eliminating the unknown sample serial dilution;
4) Inserting only one known dose of the calibrator (e. g. 500 copies).

CLAIMS

1. A method for the quantitative detection of a nucleic acid (target) from a sample, which comprises the following steps:

a) extraction of the nucleic acid from the sample with another nucleic acid (calibrator) previously added to the sample itself, said calibrator having the same sequence of the target nucleic acid, with the exception of one region which in the target nucleic acid hybridizes with a probe labeled with a reporter and a quencher, that region of the calibrator, with respect to the corresponding region of the target nucleic acid, having the same nucleotide composition, but with a random sequence, and a similar Tm,

b) mixing the extracted target nucleic acid and calibrator with primers (forward and reverse) annealing to the corresponding regions on the calibrator and on the target nucleic acid, with probes bearing a reporter and a quencher and annealing to the target nucleic acid and to the corresponding randomized region on the calibrator, and with a nucleic acid polymerase with 5'-3' nuclease activity, in suitable conditions to carry out a polymerization reaction, and

c) determination of the signal associated with the reporters released due to the 5' polymerase nuclease activity.

2. A method for the quantitative detection of a nucleic acid (target) from a sample, which comprises the following steps:

a) extraction of the nucleic acid from the sample with another nucleic acid (calibrator) previously added to the sample itself, said calibrator having the same sequence of the target nucleic acid, with the exception of those regions which in the target nucleic acid hybridize with a probe labeled with a reporter and a quencher, and additionally hybridizing with two or more

primers, said regions having each other the same nucleotide composition, but with a random sequence, and a similar Tm,

b) mixing the extracted target nucleic acid and calibrator with primers (forward and reverse) annealing to the target nucleic acid and to the corresponding randomized regions on the calibrator, with probes bearing a reporter and a quencher and annealing to the target nucleic acid and to the corresponding randomized region on the calibrator, and with a nucleic acid polymerase with 5'-3' nuclease activity, in suitable conditions to carry out a polymerization reaction, and

c) determination of the signal associated with the reporters released due to the 5' polymerase nuclease activity.

3. Method according to claims 1-2, wherein the calibrator Tm is comprised in the ±4°C range of the target nucleic acid Tm.

4. Method according to claims 1-3, wherein the 5' end of the probes is 1 to 30 nucleotides from the 3' end of the forward primer.

5. Method according to claims 1-4, wherein the probes have the 3' end blocked in order to prevent the extension by the polymerase.

6. Method according to claims 1-5, wherein said nucleic acids, said probes and said primers are DNA sequences, and the nucleic acid polymerase is thermostable DNA polymerase with 5'-3' nuclease activity.

7. Method according to claims 1-6, wherein the probes have a Tm higher than that of the primers.

8. Method according to claim 7, wherein said probes include 18 to 30 nucleotides.

9. Method according to claims 1-8, wherein said probes include a quencher label able to reduce or to avoid the reporter label fluorescence when the probes are free in solution.

10. Method according to any of the preceding claims, wherein the target nucleic acid is genomic nucleic acid of the viruses HHV-6, HHV-7, HHV-8, HIV-1 and CAMV.

11. Method according to claim 10, wherein the virus is HHV-6, the forward primer has the sequence 5' CAAAGCCAAATTATCCAGAGCG 3', the reverse primer the sequence 5' CGCTAGGTTGAGGATGATCGA 3', the target nucleic acid probe the sequence 5' CACCAGACGTCACACCCGAAGGAAT 3', and the calibrator probe the sequence 5' TACGCAACGCCAACAGACCTAGCGA 3'.

12. Method according to claim 11, wherein the calibrator is additionally randomised in the regions annealing to primers having the sequences 5' CCGGAAACCGAACATTACTGAA 3' (forward) and 5' TTACGTGAGGATGATCGAGGC 3' (reverse).

13. Method according to claim 10, wherein the virus is HHV-7, the forward primer has the sequence 5' AGCGGTACCTGTAAAATCATCCA 3', the reverse primer the sequence 5' AACAGAAACGCCACCTCGAT 3', the target nucleic acid probe the sequence 5' ACCAGTGAGAACATCGCTCTAACTGGATCA 3', and the calibrator probe the sequence 5' TAAGCCCTGACCGCACGGGTATAATACTAA 3'.

14. Method according to claim 10, wherein the virus is HHV-8, the forward primer has the sequence 5' GTCCAGACGATATGTGCGC 3', the reverse primer the sequence 5' ACTCCAAAATATCGGCCGG 3', the target nucleic acid probe the sequence 5' CATTGGTGGTATATAGATCAAGTTCCGCCA 3', and the calibrator probe the sequence 5' ACTATTCCATGCGGAATTCGAGCATAGTTG 3'.

15. Method according to claim 10, wherein the virus is HIV-1, the

forward primer has the sequence 5' TACTGACGCTCTCGCACC 3', the reverse primer the sequence 5' TCTCGACGCAGGACTCG 3', the target nucleic acid probe the sequence 5' ATCTCTCTCCTTCTAGCCTCCGCTAGTCAA 3', and the calibrator probe the sequence 5'ACTCTCAGCGGCATTCTCCTCACTTCTACT 3'.

16. Method according to claim 10, wherein the virus is CAMV, the forward primer has the sequence 5' GTCTTGCGAAGGATAGTGGGA 3', the reverse primer the sequence 5' CACGTCTTCAAAGCAAGTGGA 3', the target nucleic acid probe the sequence 5' TGCGTCATCCCTTACGTCAGTGGAGAT3', and the calibrator probe the sequence 5'ATCGCTACATGCTAGGCATCTGTGTGC 3'.

17. Use of a calibrator, as defined in the preceding claims, in a method for the quantitative detection of a nucleic acid sample.

18. Kit for the quantitation of a nucleic acid from a sample, comprising one or more calibrators, a probe specific for each target nucleic acid and a probe specific for the calibrator, two or more primers and a thermostable nucleic acid polymerase with 5'-3' nuclease activity.

Fig. 1

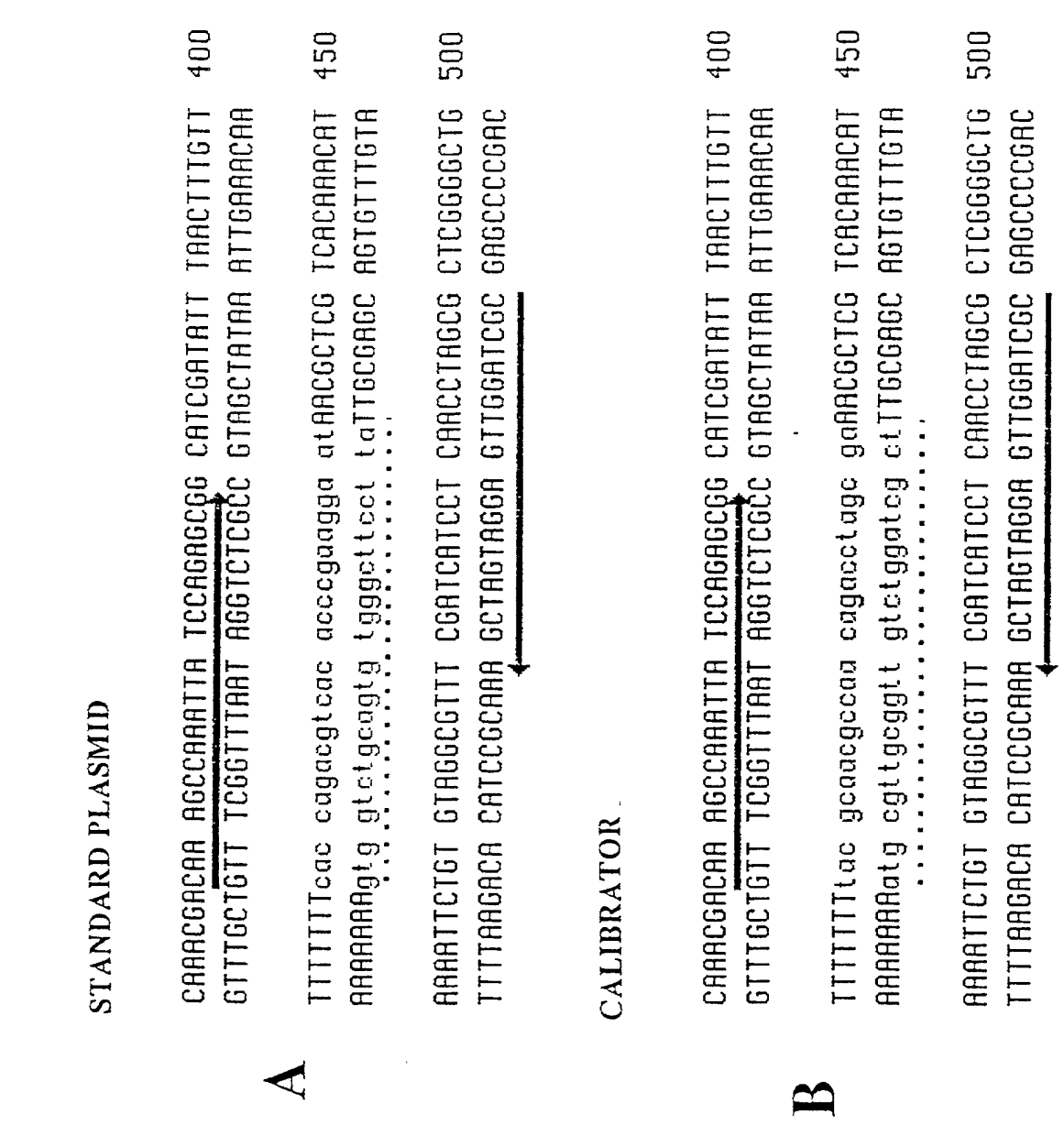

STANDARD PLASMID
A
CAAACGACAA AGCCAAATTA TCCAGAGCGG CATCGATATT TAACTTTGTT 400
GTTTGCTGTT TCGGTTTAAT AGGTCTCGCC GTAGCTATAA ATTGAAACAA
TTTTTTtac cagacgtcac accgaagga atAACGCTCG TCACAAACAT 450
AAAAAAAtg gtctgcagtg tggcttcct taTTGCGAGC AGTGTTTGTA
AAAATTCTGT GTAGGCGTTT CGATCATCCT CAACCTAGCG CTCGGGGCTG 500
TTTTAAGACA CATCCGCAAA GCTAGTAGGA GTTGGATCGC GAGCCCCGAC
CALIBRATOR
B
CAAACGACAA AGCCAAATTA TCCAGAGCGG CATCGATATT TAACTTTGTT 400
GTTTGCTGTT TCGGTTTAAT AGGTCTCGCC GTAGCTATAA ATTGAAACAA
TTTTTTtac gcaacgccaa cagacctagc gaAACGCTCG TCACAAACAT 450
AAAAAAAatg cgttgcggtt gtctggatcg ctTTGCGAGC AGTGTTTGTA
AAAATTCTGT GTAGGCGTTT CGATCATCCT CAACCTAGCG CTCGGGGCTG 500
TTTTAAGACA CATCCGCAAA GCTAGTAGGA GTTGGATCGC GAGCCCCGAC
60

Fig. 2

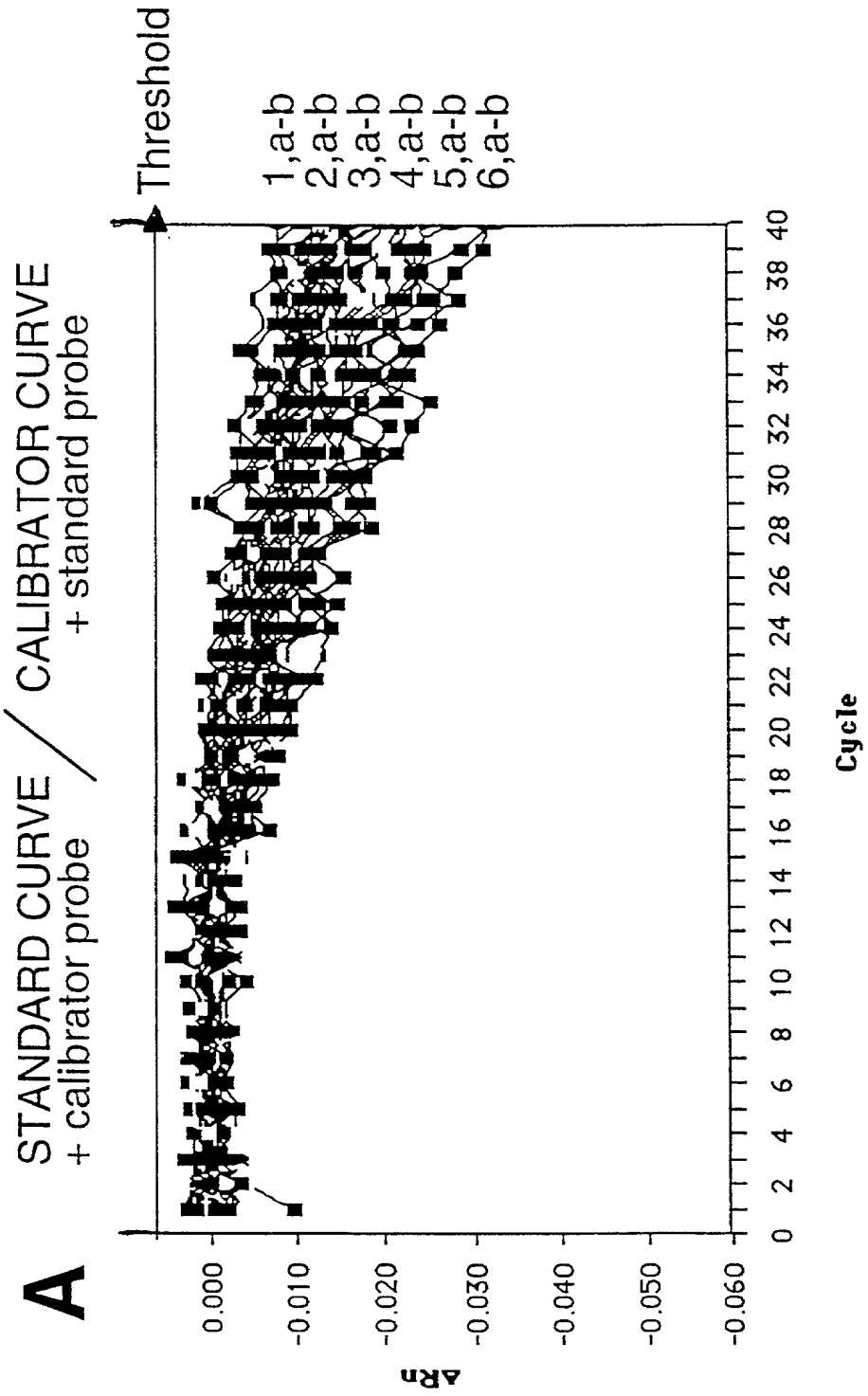


Fig. 2

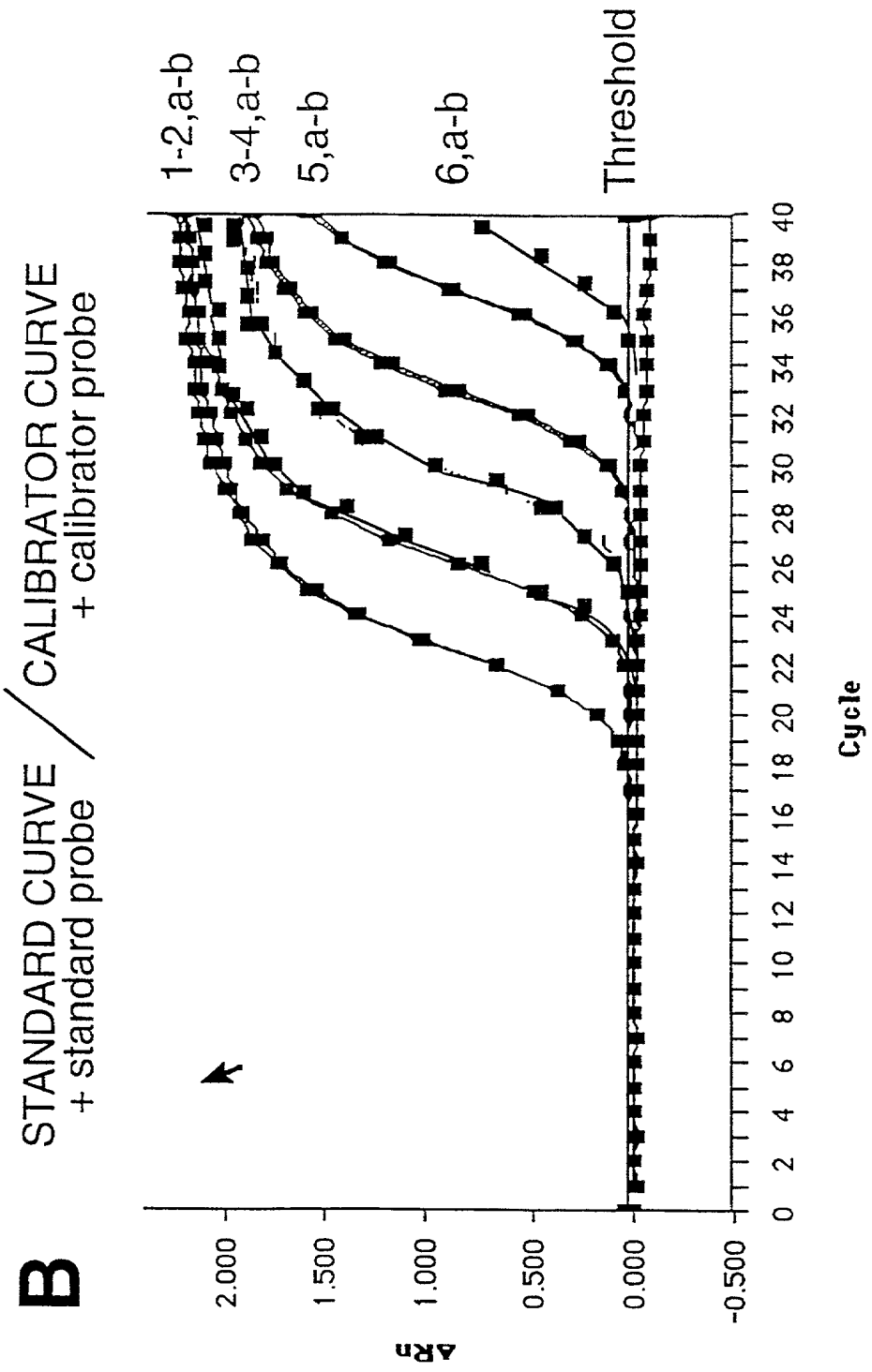

B
STANDARD CURVE
+ standard probe
CALIBRATOR CURVE
+ calibrator probe
1-2,a-b
3-4,a-b
5,a-b
6,a-b
Threshold
ΔRn
2.000
1.500
1.000
0.500
0.000
-0.500
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

Fig. 3

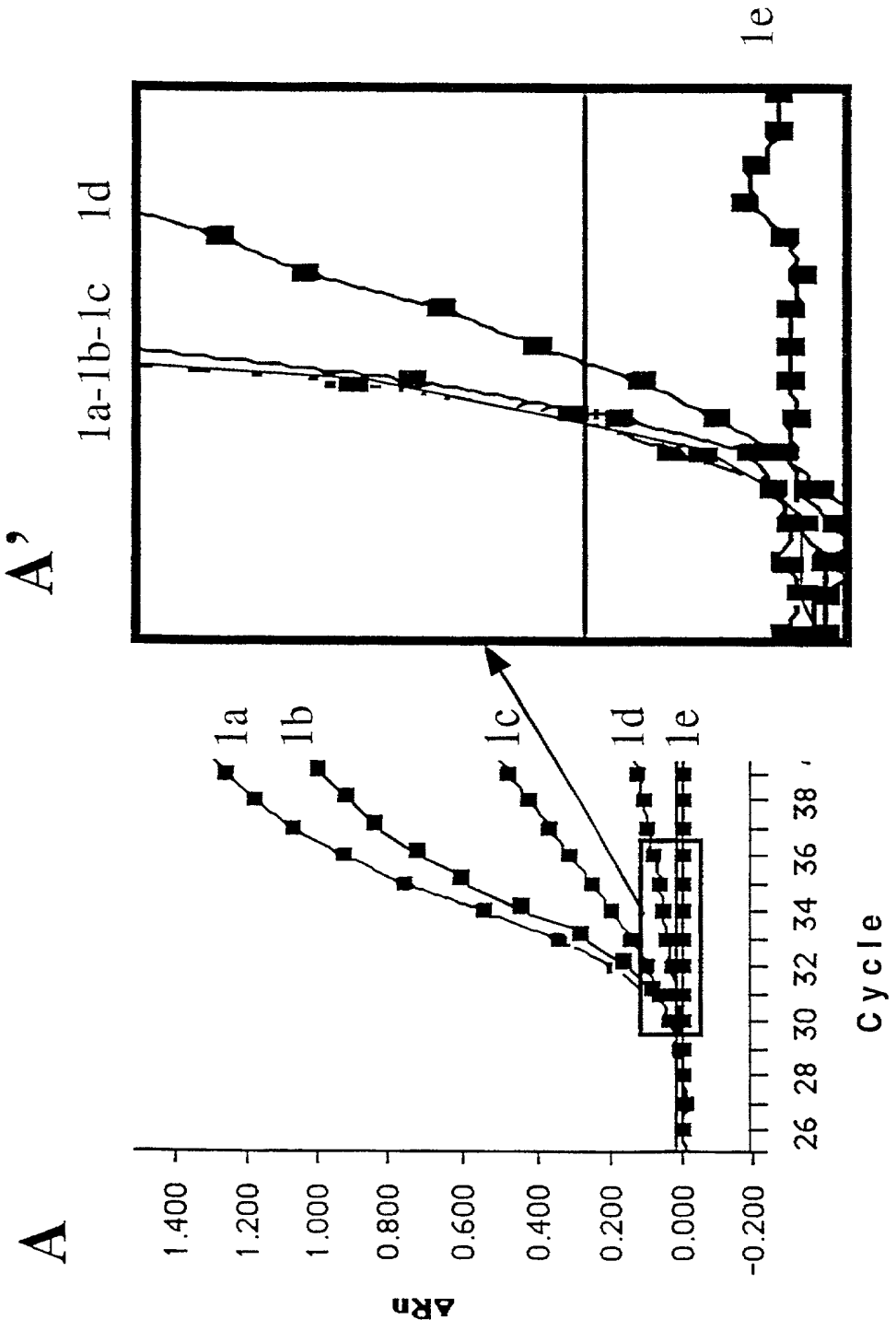

A'
1a-1b-1c 1d
1e
A
ΔRn
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
1a
1b
1c
1d
1e
26 28 30 32 34 36 38
Cycle

Fig. 3

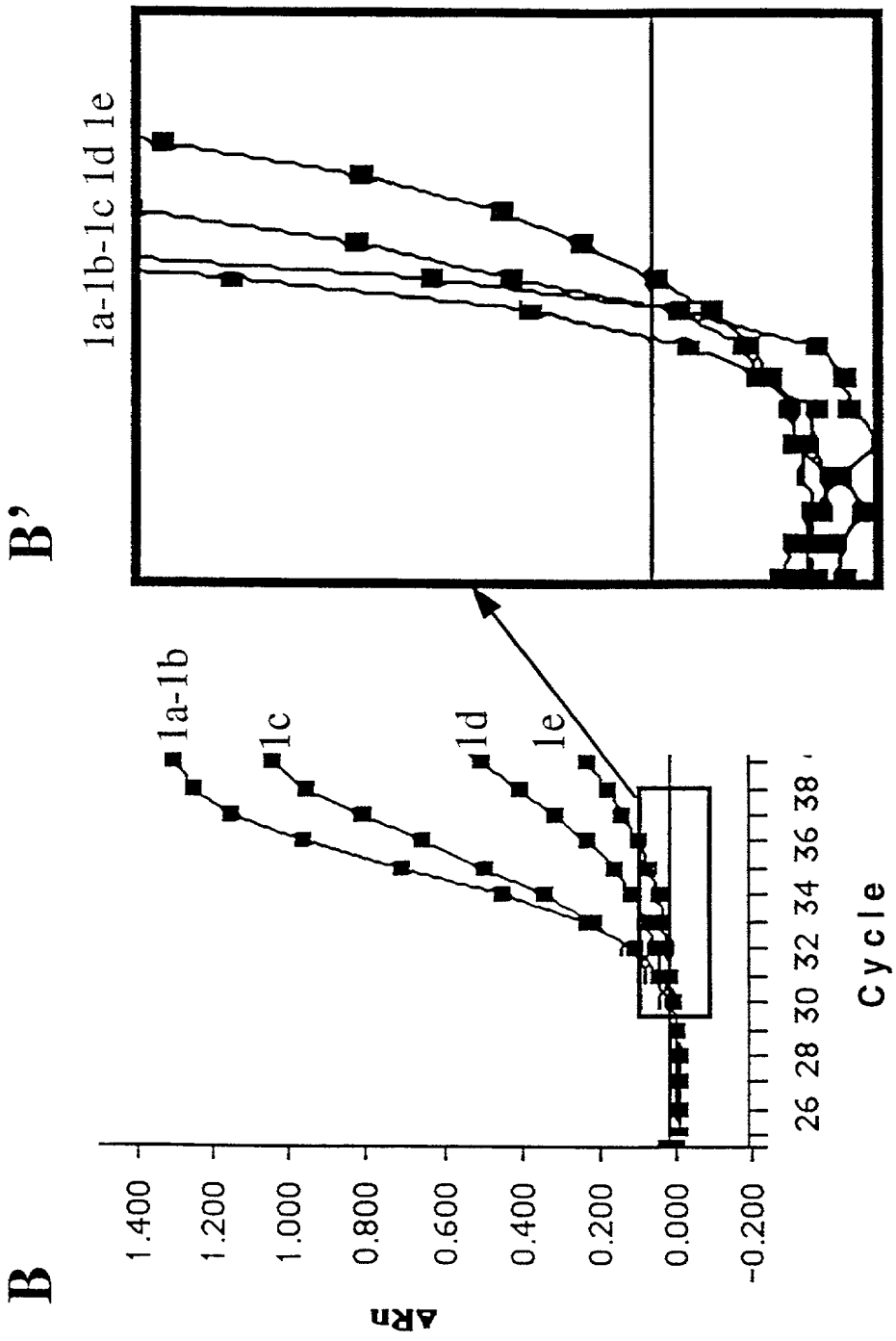
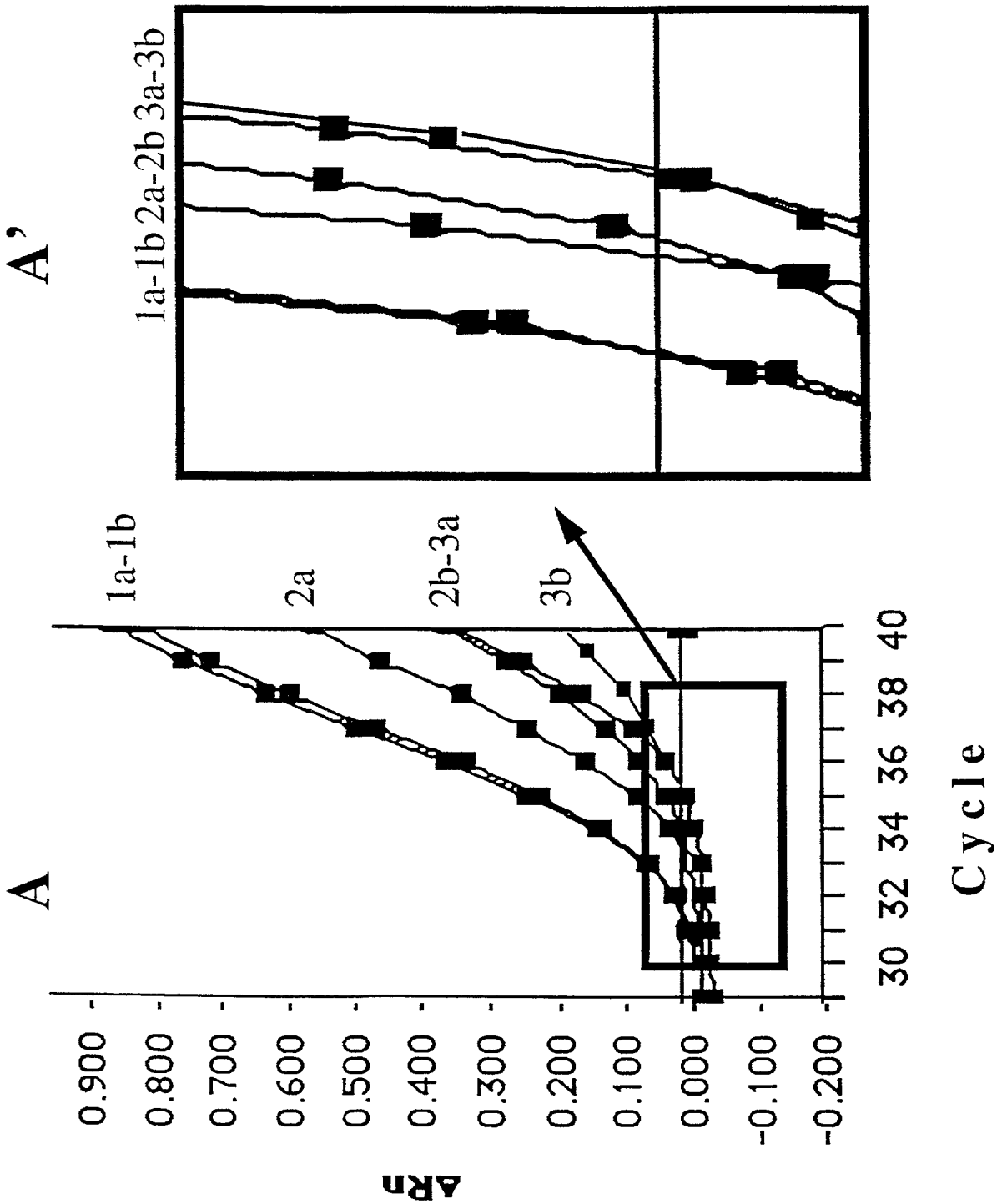

B'
1a-1b-1c 1d 1e
B
1a-1b
1c
1d
1e
ΔRn
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
-0.200
26 28 30 32 34 36 38
Cycle

Fig. 4


A
A'
ΔRn
Cycle
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
-0.200
30 32 34 36 38 40
1a-1b
2a
2b-3a
3b
1a-1b 2a-2b 3a-3b

8988 V

Case No. _____.

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method for the quantitative detection of nucleic acids

the specification of which (**check** applicable **box(s)**):

☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____
x was filed as PCT International application No. **PCT/EP99/08847** on **17.11.1999**
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s):

Application Number	**Country**	**Day/Month/Year Filed**
MI98A002491	**Italy**	**17.11.1998**

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	**Date/Month/Year Filed**

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	**Day/Month/Year Filed**	**Status: patented pending, abandoned**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, ~~25327;~~ Larry S. Nixon, 25640; ~~Robert~~ A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, ~~22770;~~ Mark E. Nusbaum, ~~32348;~~ Michael J. Keenan, ~~32106;~~ Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, ~~33149; H.~~ Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, ~~33489;~~ Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

1. Inventor's Signature: _____ Date: 25.05.2001
Inventor: ~~Giuseppe~~ (first) | MI | ~~LOCATELLI~~ (last) | Italian (citizenship)
Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via Olgettina, 58 – MILANO, Italy
(Zip Code) 20132

2. Inventor's Signature: _____ Date: 25.05.2001
Inventor: ~~Paolo~~ (first) | MI | ~~LUSSO~~ (last) | Italian (citizenship)
Residence: (city) ~~Milano~~ (state/country) Italy ITX
Post Office Address: Via Olgettina, 58 – MILANO, Italy
(Zip Code) 20132

3. Inventor's Signature: _____ Date: 15.05.2001
Inventor: ~~Mauro~~ (first) | MI | ~~MALNATI~~ (last) | Italian (citizenship)
Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via Olgettina, 58 – MILANO, Italy
(Zip Code) 20132

FOR ADDITIONAL INVENTORS, check box X and attach sheet with same information and signature and date for each.

Case No. _____

Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

4. Inventor's Signature: Francesca Salvatori Date: 25.05.2001
Inventor: ~~Francesca~~ | | ~~SALVATORI~~ | Italian
(first) | MI | (last) | (citizenship)
Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via Olgettina, 58 – MILANO, Italy
(Zip Code) 20132

5. Inventor's Signature: Gabriella Scarlatti Date: 16.05.2001
Inventor: ~~Gabriella~~ | | ~~SCARLATTI~~ | Italian
(first) | MI | (last) | (citizenship)
Residence: (city) Milano (state/country) Italy ITX
Post Office Address: Via Olgettina, 58 – MILANO, Italy
(Zip Code) 20132

6. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

7. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

8. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

9. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

10. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

11. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

12. Inventor's Signature: _____ Date: _____
Inventor: _____ | _____ | _____ | _____
(first) | MI | (last) | (citizenship)
Residence: (city) _____ (state/country) _____
Post Office Address: _____
(Zip Code) _____

SEQUENCE LISTING

<110> FONDAZIONE CENTRO SAN RAFFAELE DEL MONTE TABOR

<120> METHOD FOR THE QUANTITATIVE DETECTION OF NUCLEIC ACIDS

<130> hsr

<140>
<141>

<160> 31

<170> PatentIn Ver. 2.1

<210> 1
<211> 23
<212> DNA
<213> HHV-7

<400> 1
agcggtacct gtaaaatcat cca 23

<210> 2
<211> 20
<212> DNA
<213> HHV-7

<400> 2
aacagaaacg ccacctcgat 20

<210> 3
<211> 30
<212> DNA
<213> HHV-7

<400> 3
accagtgaga acatcgctct aactggatca 30

<210> 4
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

<400> 4
taagccctga ccgcacgggt ataatactaa 30

<210> 5
<211> 19
<212> DNA
<213> HHV-8

<400> 5
gtccagacga tatgtgcgc 19

<211> 19
<212> DNA
<213> HHV-8

<400> 6
actccaaaat atcggccgg 19

<210> 7
<211> 30
<212> DNA
<213> HHV-8

<400> 7
cattggtggt atatagatca agttccgcca 30

<210> 8
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR
MODIFIED REGION

<400> 8
actattccat gcggaattcg agcatagttg 30

<210> 9
<211> 18
<212> DNA
<213> HIV-1

<400> 9
tactgacgct ctcgcacc 18

<210> 10
<211> 17
<212> DNA
<213> HIV-1

<400> 10
tctcgacgca ggactcg 17

<210> 11
<211> 30
<212> DNA
<213> HIV-1

<400> 11
atctctctcc ttctagcctc cgctagtcaa 30

<210> 12
<211> 30
<212> DNA
<213> Artificial Sequence

<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

<400> 12
actctcagcg gcattctcct cacttctact 30

<210> 13
<211> 21
<212> DNA
<213> CAMV

<400> 13
gtcttgcgaa ggatagtggg a 21

<210> 14
<211> 21
<212> DNA
<213> CAMV

<400> 14
cacgtcttca aagcaagtgg a 21

<210> 15
<211> 27
<212> DNA
<213> CAMV

<400> 15
tgcgtcatcc cttacgtcag tggagat 27

<210> 16
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

<400> 16
atcgctacat gctaggcatc tgtgtgc 27

<210> 17
<211> 19
<212> DNA
<213> Mycobacterium tuberculosis

<400> 17
aggaggagtg gcgctgatg 19

<210> 18
<211> 17
<212> DNA
<213> Mycobacterium t. 1

actcggcgag agctgcc 17

<210> 19
<211> 22
<212> DNA
<213> Mycobacterium t. 1

<400> 19
acgaggagtc gctggccgat cc 22

<210> 20
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

<400> 20
tccagcgtca ggcgtaggca gc 22

<210> 21
<211> 17
<212> DNA
<213> Mycobacterium t. 2

<400> 21
aggcgaaccc tgcccag 17

<210> 22
<211> 17
<212> DNA
<213> Mycobacterium t. 2

<400> 22
gatcgctgat ccggcca 17

<210> 23
<211> 30
<212> DNA
<213> Mycobacterium t. 2

<400> 23
tcgacacata ggtgaggtct gctacccaca 30

<210> 24
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

actacgacta cggctgcgat cgacatcgat 30

<210> 25
<211> 22
<212> DNA
<213> HHV-6

<400> 25
caaagccaaa ttatccagag cg 22

<210> 26
<211> 21
<212> DNA
<213> HHV-6

<400> 26
cgctaggttg aggatgatcg a 21

<210> 27
<211> 25
<212> DNA
<213> HHV-6

<400> 27
caccagacgt cacacccgaa ggaat 25

<210> 28
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:CALIBRATOR'S MODIFIED REGION

<400> 28
tacgcaacgc caacagacct agcga 25

<210> 29
<211> 22
<212> DNA
<213> HHV-6

<400> 29
ccggaaaccg aacattactg aa 22

<210> 30
<211> 21
<212> DNA
<213> HHV-6

<400> 30
ttacgtgagg atgatcgagg c 21

<210> 31
<211> 25
<212> DNA
<213> HHV-6

<400> 31
tacgcaacgc caacagacct agcga 25